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STAPHYLOCOCCAL ENTEROTOXIN: ROLE OF INTESTINAL IMMUNITY IN ENTEROTOXIN B INTOXICATION (U)

by

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* Contractor
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DEFENCE RESEARCH ESTABLISHMENT SUFFIELD, RALSTON, ALBERTA

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ROLE OF INTESTINAL IMMUNITY IN ENTEROTOXIN B INTOXICATION

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ABSTRACT

We have chosen the mouse as the experimental model to investigate the role of intestinal immunity in staphylococcal enterotoxins intoxication. Although resistent to enterotoxins, the mouse has a certain advantage over other models by not having an emetic mechanism, so no loss in peroral administered enterotoxin takes place. Parenteral administration of enterotoxins was observed to induce a high titer of specific antibodies in serum, mainly of IgG and IgG2a subclasses. Peroral administration of enterotoxin elicited a good response at the intestinal level as showed by specific anti-enterotoxin antibodies in the supernatants of *"in vitro"* cultured intestinal fragments and the synthesized immunoglobulin was IgA. When different routes, either single or in combinations, were studied, it was observed that a parenteral followed by peroral administration of enterotoxin induced the higher intestinal immune response. Secretion of specific anti-enterotoxin antibodies by the intestinal immune system also persisted longer than antibodies in serum of perorally immunized animals.

RÉSUMÉ

Nous avons choisi la souris comme modèle expérimental pour l'étude du rôle de l'immunité intestinale dans les intoxications dues aux endotoxines staphylococciques. Bien que résistante aux endotoxines, la souris comporte un avantage par rapport aux autres modèles du fait qu'elle ne possède pas de méchanisme hémétique, de sorte qu'il n'y a pas de perte d'entérotoxine administrée par voie orale. On a constaté que l'administration parentérale d'entérotoxines provoquait un titre plus élevé d'anticorps spécifiques dans le sérum, surtout des sous-classes IgG et IgG2a. L'administration orale d'entérotoxine a provoqué une bonne réaction au niveau intestinal comme en témoigne la présence d'anticorps anti-entérotoxine spécifiques dans les surnageants de fragments intestinaux cultivés *in vitro*; l'immunoglobuline synthétisée était une IgA. Lorsqu'on a étudié des voies d'administration différentes, seules ou en combinaison, on a constaté que l'administration parentérale d'entérotoxine suivie d'une administration orale provoquaient la plus forte réponse immunitaire intestinale. La sécrétion d'anticorps anti-entérotoxine spécifiques par le système immunitaire de l'intestin a également persisté plus longtemps que celle des anticorps sériques chez les animaux immunisés par voie orale.

INTRODUCTION

Staphylococcus aureus food poisoning is one of the most common of the reported cases of foodborne illnesses. It is caused by a closely related group of enterotoxins, which are water soluble proteins and are released into the medium during growth by certain strains of the gram-positive bacterium *Staphylococcus aureus*. These toxins are of small molecular weight (26-28 KD), single, unbranched polypeptide chains and have been differentiated by their immunological specificity into seven distinct types designated as staphylococcal enterotoxin (SE), SEA, SEB, (Casman, Bergdoll and Robinson, 1963), SEC_1 (Borja and Bergdoll, 1967) SEC_2 (Avena and Bergdoll, 1967) SEC_3 (Reiser, Robbins, Noleto, Khoe and Bergdoll, 1984) SED (Casman, Bennett, Dorsey and Issa, 1967) and SEE (Bergdoll, Borja, Robbins and Weiss, 1971).

The biological effects of a SEs intoxication are seen in a limited number of species. Humans and primates present similar symptoms i.e. vomiting and diarrhea, and in severe cases, death. Cats (Bayliss, 1940) and dogs (Kocandrle, Houttiun and Prohaska, 1966) display emetic responses to SE only after intravenous administration. Weanling pigs can elicit an emetic response following per-oral or intraduodenal administration of SEA (Taylor, Schlunz, Beery, Cliver and Bergdoll, 1962). Rabbits can develop shock symptoms after repeated intravenous, intramusculary or intradermal injection of SEs but never an emetic response. Rodents (mice, rats and guinea-pigs) are resistant to relatively high doses of SEs independent of the administration route and are devoid of emetic mechanisms (Beery, Taylor, Schlunz, Freed and Bergdoll, 1984). The absence of vomiting reactions induced by SEs in rodents might be an advantage for the study of intestinal immunity since no administered SEs are lost by vomiting. We therefore, chose mice as a model for the study of intestinal immunity to SEB.

Diverse environmental immunogens of microbial and food origin constantly stimulate the entire immune system. The SEs belong to the environmental immunogens because *S. aureus*, secreting different SEs, are frequently present on human or animal skin, wounds or food. The SEs are strong immunogens and their constant presence will, in normal subjects, continuously induce a high amount of anti-SE antibodies in the serum of normal subjects (Notermans, Van Leeuwen, Duffrenne and Tips, 1983). Mucosal membranes, which represent a vast area of contact with the environment, are exposed daily to these immunogens which may induce specific humoral as well as cell-mediated immune responses. The common route of human or animal exposure to SE has been through ingestion of preformed SEs in contaminated food. No attempt has ever been made to study the intestinal immune response to SEs. In this communication we describe the possible role of the intestine on the development of immunity to SEB.

MATERIALS AND METHODS

Animals

Inbred C57B1/6 mice of both sexes (Charles River, St. Constant, Que) 6-8 weeks of age at the onset of immunization were used. Mice were maintained on water and laboratory chow ad libitum and handled according to Canadian Council on Animal Care regulations.

Reagents

SEB was obtained from Toxin Technology Inc., (Madison, Wisconsin). The toxin was quantitated based on its extinction coefficient of $E^{1\%}_{1cm} = 14.4$ at 277 nm. All other chemical reagents used were of an alytical grade and purchased from Fisher Scientific (Montreal, Que.).

Immunization Procedures

Before forming the experimental groups, mice were tested for the presence of naturally occurring anti-SEB antibodies in their serum. About 20-28% of the total screened mice population had an acceptable low titer (less than 1/50) to be included into experiment. From these animals, groups of 6-10 mice were selected and submitted to parenteral (intraperitoneally, subcutaneously), or peroral immunization. Intraparental injections contained 10 μg of SEB/animal at day 0 and 20 μg SEB/animal 14 days later. The first injection was given with Freund complete adjuvant and the second with Freund incomplete adjuvant (Difco, Canlab Montreal, Que.). Peroral immunized animals received, via a feeding needle, the same amount of enterotoxins in phosphate-buffered saline (PBS) - 0.1M sodium bicarbonate solution in order to neutralize the acid gastric medium. Intraparental control groups

had the SEB replaced by an equivalent amount of BSA and peroral control groups were given BSA in PBS-bicarbonate solution.

Periodically, sera were obtained by bleeding the animal tails. All the obtained sera were kept frozen at -20°C until tested for the presence of anti-SEB specific antibodies.

Intestinal Antibody Synthesis

Local antibody synthesis by the intestine was investigated by culturing intestinal tissue fragments to produce antibody *in vitro*. The culture of intestinal fragments was done according to the method of Svennerholm, Lange and Holmgren (1978) with minor modifications. One month after the last peroral SEB administration the food was removed and 24 h later the mice were killed with carbon dioxide and the small intestine immediately excised. The intestine was minced and washed with PBS followed by washing with MEM-Eagle culture medium containing 5% heat inactivated normal rabbit serum and 200 IU of penicillin-streptomycin per mL. Inhibition of proteases activity in intestinal fragment cultures was done by adding 0.1 mg/mL soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) in 50 mM EDTA and 50 μg/mL of phenylmethylsulfonyl fluoride (Boehringer Mannheim, Montreal, Que.). Intestinal fragments were cultured in tubes for 24 h at 37°C in a humid CO_2 incubator. To assure comparability between different samples, 2 mL of culture medium was added for each gram of intestinal fragments. At the end of the culture period the tubes were centrifuged and the supernatants kept for anti-enterotoxin antibody determination. The measurement of the newly formed anti-enterotoxin antibodies was carried out by ELISA.

Determination of Immunoglobulin Types and Measurement of Specific Antibody Responses

Quantitative determination of immunoglobulin types present in sera of immunized animals was possible only in samples from parenteral immunized mice since the quantity of antibodies present in sera of peroral immunized animals were found to be too low to be accurately measured by the radial immunodiffusion method. The quantitative radial immunodiffusion (Mancini, Carbonara and Heremans 1965) was done using commercially available plates for mouse IgG total, IgG subclasses, IgA and IgM immunoglobulins (Milles Scientific, Rexdale, Ont.) according to the manufacturer's instructions. All other immunoglobulin types and specific anti-enterotoxin induced antibodies were determined according to one of the following two arrangements:

a. Isotypes of immunoglobulins in sera of peroral immunized mice were determined by capture type ELISA (Tijssen, 1985). Briefly, Immulon 1 microplates (Fisher Scientific, Montreal, Que.) were first coated with rabbit anti-mouse IgG (reacting with all Ig classes) (Cedarlane, Hornby, Ont.) at a previous established concentration. Plates were then incubated at 4°C overnight, washed with PBS containing 0.05% v/v Tween 20 (PBS-T) and saturated with 2% BSA. After washing, serially diluted samples were incubated for 2 h at 37°C, washed and incubated again with specific rabbit or sheep anti-mouse Ig classes antisera conjugated with horseradish peroxidase (HRP) (Cappel, Organon Teknika, Scarborough, Ont.). The enzymatic reactions were developed with ABTS substrate (Cappel, Organon Teknika, Scarborough, Ont.) and the colour read at 405 nm with an ELISA microplate reader (EL309, Biotek Instruments, Burlington, VT). All samples were determined in triplicate and control values were subtracted; or

b. Specific anti-SEB immunoglobulins were determined essentially as above, except that SEB was first coated on Immulon 2 microplates (Fisher Scientific, Montreal, Que.).

Statistical Analysis

The Students t test for statistically significant difference (P) for two independent means i.e. values for immunized and control animals were used.

RESULTS

Type and Quantitation of Immunoglobulins in Sera of Mice Parenterally Immunized with SEB

A group of six mice were in munized with two doses of SEB (10 μg and 20 μg/animal) and individual serum was quantified for immunoglobulin classes and subclasses by the radial immunodiffusion technique. Compared to control animals, our results showed an increase in the class of IgG, mainly in the IgG_1 and IgG_{2a} subclasses. Small increases noted in immunoglobulin IgM and IgA classes were not significant. Although this method did not quantitate specific antibodies to SEB, the overall increases of IgG class can only be attributed to an immunological response to injected enterotoxins (Table 1).

Immunoglobulin Types in Sera of Peroral Immunized Mice with SEB

Immunoglobulin types, found in the sera of mice following peroral administration of SEB were identified by capture ELISA. The results presented in Table 2 show that a limited rise in immunoglobulins of serum was observed in peroral immunized mice and that the only significant increase were the immunoglobulins of IgG type.

Specific Anti-SEB Immunoglobulin Synthesis by Intestinal Fragments from Parental and Peroral Immunized Mice

In these experiments we studied the specific immune response at the intestinal level for two routes of SE administration: parental (intraperitoneally) and peroral. Supernatants, from intestinal fragment cultures from intraperitoneally immunized mice were reacted on microplates coated with SEB and the isotype determined with class-specific anti-mouse antisera conjugated with HRP. Groups of 10 mice were used for this experiment. Results presented in Table 3 show that a small non-significant increase was observed in the specific anti-SEB response for classes IgG and IgM. However, a significant increase at $P<0.01$ was observed for specific IgA anti-SEB antibodies. This showed that the parental route affected the local immune response by stimulating IgA producing cells to secrete specific antibodies.

In contrast with the above results, intestinal fragment cultures from peroral immunized mice contained specific anti-SEB antibodies in higher titer (Table 4). These results showed an active immunoglobulin synthesis at the intestinal level following peroral immunization. As common for mucosal secretions the main immunoglobulin synthesized was IgA. In another set of experiments we found that peroral administration of SEB induced a prolonged intestinal response to SEB as compared to the presence of anti-SEB antibodies in serum induced by the same peroral administration route (Table 5). These results further demonstrate that peroral SEB administration can efficiently activate the local immune response.

Role of Intravenous SEB Administration on the Induction of Intestinal Response

Mice immunized with one or two 10 μg doses of SEB, did not develop any intestinal immunity since no immunoglobulin synthesis could be detected in the

supernatants of cultured intestinal fragments. However, after the administration of the third SEB dose, specific anti-SEB antibodies could be measured. However, the increase was limited only to the IgA class and statistical calculation did not give a significant result (data not shown).

Combination of Two Administration Routes on the Induction of Intestinal Immune Response

In the first experiment, mice were immunized with SEB by the intraperitoneal route in association with Freund's complete adjuvant, followed after 14 days by a second dose of SEB administered orally. In the second experiment, the order of routes was reversed. The intestinal immune response was followed, as in previous experiments, by the synthesis of specific anti-SEB antibodies by "*in vitro*" cultured intestinal fragments.

The results presented in Tables 6 and 7 showed that the sequence of routes for SEB administration was important. A much higher intestinal immune response was observed when first SEB was given to animals by parenteral route followed by peroral route.

DISCUSSION

Enterotoxins can induce food poisoning, an illness of short duration with severe symptoms. In spite of the severity of the disease, immunization against SEB is not recommended since no absolute proof exist that anti-SEB antibodies can protect people.

This type of investigation is compromised by a lack of a suitable animal model for information on the role of intestinal immunity to SEs. In general, it was observed that under the experimental conditions we used, SEB acts on the local

immune response similar to that observed with other antigens such as different proteins or toxins (Mestecky, 1987). Mice immunized by the parenteral route developed high titers of antibodies in serum, especially of the IgG class (with the IgG_{2a} subclass being preferencially induced (Table 1). These animals also developed some intestinal immunity as observed in intestinal fragment cultured from parenterally immunized animals (Table 3). However, any quantity of immunoglobulins produced must be less than the 1 μg level since these could not be determined by quantitative radial immunodiffusion technique (sensitivity 1-3 μg immunoglobulin/mL). When compared by ELISA method (sensitivity 5-10 ng immunoglobulin/mL), parenterally immunized animals showed an apparent increase in local synthesis for all classes, the IgA class being statistically significant. As expected, since IgA class is the major immunoglobulin related to intestinal immunity.

On the other hand, peroral immunization induced a significant increase in all classes of immunoglobulins. Once again the IgA class showed by far the higher increase (Table 4). Peroral SEB administration can also, to a limited extent, induce the appearance of specific anti-SEB antibodies in serum (Table 2). This can, in part, explain the presence of anti-SEB antibodies in human serum which might have been induced by ingestion of food contaminated with SEs over a life time period (Notermans *et. al.*, 1983).

It has been shown that rabbits, primed with *Shigella* organisms intragastrically, displayed a marked increase in specific antibody production in isolated intestinal loops after subsequent oral administation of these bacteria (i.e. a memory response) (Keren *et. al.*, 1982; Keren *et. al.*, 1985). On the other hand, a parental priming was necessary before obtaining a secondary response with oral administered antigen (Pierce and Gowans, 1975). With cholera antigen preparations (toxin β sub-unit) Svennerholm *et. al.*, (1982) have shown that administration of antigen either orally or intramuscularly can induce IgA antitoxin response in intestinal secretions. That systemic presentation of cholera toxin can stimulate

intestinal immunity (Svennerholm et al., 1978) is shown in our own results where administration of SEB intraperitoneally in mice can induce a specific IgA response in supernatants of cultured intestinal fragaments (Table 7). Similar to our findings, Svennerholm *et. al.*, (1982), have reported that antitoxin response in serum did not last as long as the intestinal antitoxin response seen after oral immunization (Table 5).

When the efficacy of SEB administration routes were compared, we observed that a first parenteral and a second peroral administration was most suitable for induction of a high intestinal immune response (Table 6). These results are comparable with experiments in rats immunized with cholera toxin (Pierce, 1984).

Our results suggest that enterotoxins induced a marked local intestinal immune response which along with other mouse physiological properties can explain the resistance of this species to SEs. In the absence of a reliable animal model, all extrapolation to human intestinal immunity to SEB remain speculative as is the physiopathology of SEs, but our results are encouraging.

The development for a human vaccine against SE is still distant. For obvious reasons peroral vaccination can not be done in humans with native toxins. However, in our opinion much more research has to be done, focussed perhaps, on:

a. Identification of the toxic site of SE molecules either by the use of synthetic peptides or by alteration of genes of SEB producing *S. aureus* strains, in order to sequentionally reduce the size of the molecule, thus delimiting the toxic site; and

b. Identification of possible receptors for SEB at the intestinal level. Although no receptors for SE were found on Henle 407 embryonic human intestinal epithelial cell line (Buxser *et. al.*, 1981) the work of

Reck *et. al.*, (1988) with anti-idiotypic antibodies to SEB implies the existence of receptors for SEB on the intestinal membrane. If the binding site of SEB on intestinal membrane is different from the toxic site, then it might be possible to block those receptors with synthesized peptides corresponding to the binding region.

In conclusion, although considerable efforts have been made by others to understand the physiopathology of SEB, and although very little information is available on the mode of cellular site of toxin action in the gastrointestinal tract, our results are encouraging in that our experimental conditions enhanced the intestinal IgA response in mice, and may lead to enhanced immunity against SEs for humans.

TABLE 1

TYPE AND QUANTITATION OF IMMUNOGLOBULINS IN SERA OF MICE PARENTERALLY IMMUNIZED WITH SEA*

Immunization Schedule	Type of Ig						
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgG (total)	IgA	IgM
Day 0 Control	1.30±0.65	3.14±0.85	0.61±0.45	0.30±0.40	5.20±0.55	0.18±0.35	1.10±0.10
Day 0 experimental	1.20±0.80	3.68±0.75	0.60±0.52	0.40±0.25	5.30±0.65	0.20±0.15	1.00±0.10
Day 7 after immunization	1.32±0.55	3.25±0.78	0.63±0.40	0.42±0.30	5.35±0.45	0.21±0.20	1.05±0.20
Day 14 after immunization	3.30±0.45	8.20±0.65**	1.05±0.62	0.50±0.30	13.85±0.35 **	0.35±0.20	1.35±0.35

* mean and standard deviation of six animals/groups (mg/ml)

** $P<0.001$

TABLE 2

ELISA RECIPROCAL TITERS* OF SERUM SAMPLES OF MICE ORALLY IMMUNIZED WITH SEB

Antibody Isotype	Relative Antibody Quantities	
	Control	SEB Immunized**
IgG	0.82 ± 1.02	$3.20 \pm 1.03^{**}$
IgA	0.04 ± 1.10	1.65 ± 1.58
IgM	0.88 ± 0.85	2.00 ± 1.33

* mean and standard deviation of 10 mice/group
** results significant for $P<0.001$

TABLE 3

ELISA RECIPROCAL TITERS* OF SUPERNATANTS FROM "IN VITRO" CULTURED INTESTINAL FRAGMENTS OF MICE PARENTALLY IMMUNIZED WITH SEB

Antibody Isotype	Relative Antibody Quantities	
	Control	SEB Immunized**
IgG	0.92±0.65	1.30±0.62
IgA	1.05±0.45	2.42±0.47**
IgM	0.93±0.72	1.55±0.85

* mean and standard deviation
** results significant for $P<0.001$

TABLE 4

ELISA RECIPROCAL TITERS* OF SUPERNATANTS FROM "IN VITRO" CULTURED INTESTINAL FRAGMENTS OF MICE IMMUNIZED PERORAL WITH SEB

Antibody Isotype	Relative Antibody Quantities	
	Control	SEB Immunized**
IgG	0.94±1.10	4.20±1.75
IgA	0.85±1.11	8.00±3.26
IgM	1.10±0.96	2.20±1.86

* mean and standard deviation
** results significant for $P<0.001$

TABLE 5

PERSISTENCE OF SPECIFIC ANTI-SEB ANTIBODIES IN SERUM AND SUPERNATANTS OF INTESTINAL FRAGMENTS IN MICE IMMUNIZED BY ORAL ROUTE

		DAY				
		0	7	14	21	28
Serum	IgG	0.82±0.75**	2.40±1.65	3.62±1.56	2.78±1.82±	2.24±1.94
Intestinal Fragments	IgG	1.08±0.92	3.20±1.12	nd	2.20±1.68	0.85±0.80
	IgA	0.05±0.40	12.10±4.80	nd	10.50±2.62	8.80±2.20
	IgM	0.80±1.10	4.10±1.40	nd	2.90±1.85	1.00±0.91

* ELISA reciprocal titers

** mean and standard deviation from 15 animals

nd not done

TABLE 6

ELISA RECIPROCAL TITERS* OF SUPERNATANTS FROM "IN VITRO" CULTURED INTESTINAL FRAGMENTS OF MICE IMMUNIZED WITH SEB INTRAPERITONEALLY FOLLOWED BY A PERORAL BOOST

Antibody Type	Relative Antibody Quantities	
	Control	SEB Immunized**
IgG	1.10 ± 0.96	3.75 ± 1.80**
IgA	1.40 ± 0.85	11.75 ± 2.88**
IgM	0.94 ± 0.92	1.62 ± 1.05

* mean and standard deviation
** results significant for $P0<0.001$

TABLE 7

ELISA RECIPROCAL TITERS* OF SUPERNATANTS FROM "IN VITRO" CULTURED INTESTINAL FRAGMENTS OF MICE IMMUNIZED WITH SEB INTRAPERITONEALLY FOLLOWED BY AN INTRAPERITONEALLY BOOST

Antibody Type	Relative Antibody Quantities	
	Control	Immunized**
IgG	0.95 ± 0.87	2.80 ± 1.72**
IgA	1.15 ± 1.05	7.86 ± 2.88**
IgM	1.00 ± 1.00	1.75 ± 1.10

* mean and standard deviation
** results significant for $P<0.001$

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To investigate the role of intestinal immunity in staphylococcal enterotoxins intoxication. We have chosen the mouse as experimental model, which although resistent to enterotoxins, has a certain advantage by not having an emetic mechanism, thus no loss in peroral administered enterotoxin takes place. Parenteral administration of enterotoxins induced a high titer of specific antibodies in serum, mainly of IgG and IgG2a subclasses. Peroral administration of enterotoxin elicited a good response at the intestinal level as showed by specific anti-enterotoxin antibodies in the supernatants of "in vitro" cultured intestinal fragments and the synthesized immunoglobulin was IgA. When different routes and combinations of them were studied it was observed that a parenteral followed by peroral administration of enterotoxin induced the higher intestinal immune response. Secretion of specific anti-enterotoxin antibodies by intestinal immune system also lasted longer compared to the presence of antibodies in serum of perorally by immunized animals.

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Staphylococcus aureus

Enterotoxin B

IgA

Immunity